

# Silencing Nociceptor Neurons Reduces Allergic Airway Inflammation

## Highlights

- Nociceptor sensory neuron ablation reduces allergic airway inflammation
- QX-314 enters through large-pore ion channels to silence airway sensory neurons
- IL-5 triggers sensory neuron release of peptides that drive immune cell responses
- Silencing nociceptors is a new strategy to treat type 2 inflammation and allergies

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## In Brief

Talbot et al. provide mechanistic insights into how neuro-immune interplay amplifies type 2 allergic airway inflammation. Based on this, they propose a new treatment strategy for asthma, using charged sodium channel blockers to selectively silence sensory neurons in the lung.



# Silencing Nociceptor Neurons Reduces Allergic Airway Inflammation

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## SUMMARY

Lung nociceptors initiate cough and bronchoconstriction. To elucidate if these fibers also contribute to allergic airway inflammation, we stimulated lung nociceptors with capsaicin and observed increased neuropeptide release and immune cell infiltration. In contrast, ablating Nav1.8<sup>+</sup> sensory neurons or silencing them with QX-314, a charged sodium channel inhibitor that enters via large-pore ion channels to specifically block nociceptors, substantially reduced ovalbumin- or house-dust-mite-induced airway inflammation and bronchial hyperresponsiveness. We also discovered that IL-5, a cytokine produced by activated immune cells, acts directly on nociceptors to induce the release of vasoactive intestinal peptide (VIP). VIP then stimulates CD4<sup>+</sup> and resident innate lymphoid type 2 cells, creating an inflammatory signaling loop that promotes allergic inflammation. Our results indicate that nociceptors amplify pathological adaptive immune responses and that silencing these neurons with QX-314 interrupts this neuro-immune interplay, revealing a potential new therapeutic strategy for asthma.

## INTRODUCTION

Asthma is an inflammatory airway disease caused by environmental (allergens, air pollution, cold, and smoking) and genetic interactions (Martinez, 2007). The disease affects 7%–10% of the world's population, causes ~250,000 deaths annually (Akinbami, 2006), and its prevalence is increasing (Ramsey and Celedón, 2005). Asthma symptoms include wheezing, coughing, chest tightness, and shortness of breath, caused by increased airway hyperresponsiveness, inflammation, mucus hypersecretion, and structural remodeling (Locksley, 2010). Histopathology

shows goblet cell metaplasia, thickened basement membranes, increased airway smooth muscle, and inflammatory cell infiltration (Locksley, 2010). Immune cells, particularly innate lymphoid type 2 cells (ILC2), T helper 2 (Th2) cells, and eosinophils, are central to the pathological airway transformation. Inhaled allergens, such as house dust mites (HDMs), viruses, or bacteria, are sensed mainly by dendritic cells in the lung mucosa, which promote precursor Th cell differentiation into Th2 cells. These, along with ILC2 cells, initiate an inflammatory response that includes recruitment and activation by cytokines of immune effector cells, with eosinophils contributing to bronchoconstriction, microvascular permeability, and airway remodeling (Kumar et al., 2005; Locksley, 2010).

The lung is densely innervated by sensory fibers, most of which express markers of nociceptors, including the transient receptor potential (TRP) channels TRPV1 and TRPA1 (Ni et al., 2006). Airway nociceptors respond to chemical, mechanical, or thermal stimuli to initiate essential protective airway reflexes, such as coughing (Canning et al., 2006). Asthmatic patients have a denser network of these fibers around small airways (Barnes, 1996; Myers et al., 2002) and a reduced activation threshold in response to airborne irritants (Canning and Spina, 2009). Patients also display elevated neuropeptide levels in bronchoalveolar lavage fluids (BALFs) (Lilly et al., 1995). These features indicate changes in and excess activity of peptidergic sensory fibers (Patterson et al., 2007). The large-pore cation channels TRPV1 and TRPA1 are activated by exogenous chemical irritants, such as cigarette smoke (Kanezaki et al., 2012), and also directly and indirectly via GPCR- and receptor tyrosine kinase coupling by many endogenous ligands generated during inflammation, including protons, lipids, endogenous cannabinoids, bradykinin, and NGF (Szallasi et al., 2007). Stimulation of nociceptor peripheral terminals results in calcium-mediated vesicular release of neuropeptides, like substance P and calcitonin gene-related peptide (CGRP), to generate neurogenic inflammation, which is characterized by increased vascular permeability and vasodilation. This is amplified and spreads by the antidromic reflex, where the sensory input in one branch of a sensory neuron initiates an action potential back down a

connecting branch to its peripheral terminal (Chiu et al., 2012). Nociceptors may contribute to airway disease both by their capacity to produce bronchoconstriction (Tränkner et al., 2014) and local neurogenic inflammation (Caceres et al., 2009; Hox et al., 2013). Supporting involvement of sensory fibers in key aspects of type 2 inflammation, genetic knockout (KO), or pharmacological antagonism of the TRPA1 channel reduced inflammation in a mouse model of allergic airway disease (Caceres et al., 2009), while ablation of TRPV1 afferents blocked bronchial hyperresponsiveness (Tränkner et al., 2014). However, exactly how sensory neurons and immune cells cooperate to amplify immunopathology and direct various types of inflammation is unknown (Chiu et al., 2013; Liu et al., 2014; Nussbaum et al., 2013; Riolo-Blanco et al., 2014; Wilson et al., 2013).

We have investigated the contribution of lung nociceptor neurons to the generation and resolution of allergic airway inflammation by asking if global ablation of all nociceptor neurons (Na<sub>v</sub>1.8-expressing neurons) attenuates allergen-induced immune responses. We also temporarily silenced nociceptors using inhaled permanently charged quaternary derivatives of sodium channel blockers (Binshtok et al., 2007). The charge of these quaternary compounds prevents their diffusion through neuronal membranes, making them ineffective as local anesthetics. However, their small size enables them to permeate into cells through activated large-pore channels, including TRPV1 (Binshtok et al., 2007) and TRPA1 (Lennertz et al., 2012). A combination of a TRP channel opener and the charged sodium blocker QX-314 produces prolonged (>9 hr) peripheral nociceptor silencing and a pain- or itch- specific local blockade (Binshtok et al., 2007; Brenneis et al., 2013; Roberson et al., 2013). This strategy enabled us to determine the consequences of blocking lung nociceptor activity on established allergic inflammation. We found that sensory neurons, in part through the vasoactive intestinal peptide (VIP)-VPAC2 axis, drive CD4<sup>+</sup> and ILC2 cell production of cytokines including IL-5, and reciprocally that IL-5 activates sensory neurons. Our results indicate that nociceptors play a major role in amplifying adaptive immune responses in the lung, and they show that local pharmacological silencing of nociceptors interrupts the pro-inflammatory signaling loop between neurons and immune cells to enhance allergic airway inflammation resolution.

## RESULTS

### Nociceptor Activation and Allergic Airway Inflammation

Allergic inflammation and bronchial hyperresponsiveness can be induced in mice by an initial sensitization to an allergen, in this case ovalbumin (OVA) (intraperitoneally [i.p.], days 0 and 7), followed by inhaled OVA challenges (days 14–17) (Figure 1A). To investigate if sensory fibers play a role in allergic airway inflammation, we first performed lung nociceptor gain-of-function experiments *in vivo*. Mice were treated with intranasal instilled capsaicin, a selective TRPV1 agonist (Szallasi et al., 2007), and 24 hr later BALF was immunophenotyped. In naive mice (no OVA exposure), capsaicin treatment increased CD45<sup>+</sup> cells (marker of all immune cells) (Figure 1B). In OVA-treated mice, capsaicin administration on day 18 (the day after completing OVA challenges and time of maximal inflammation) further

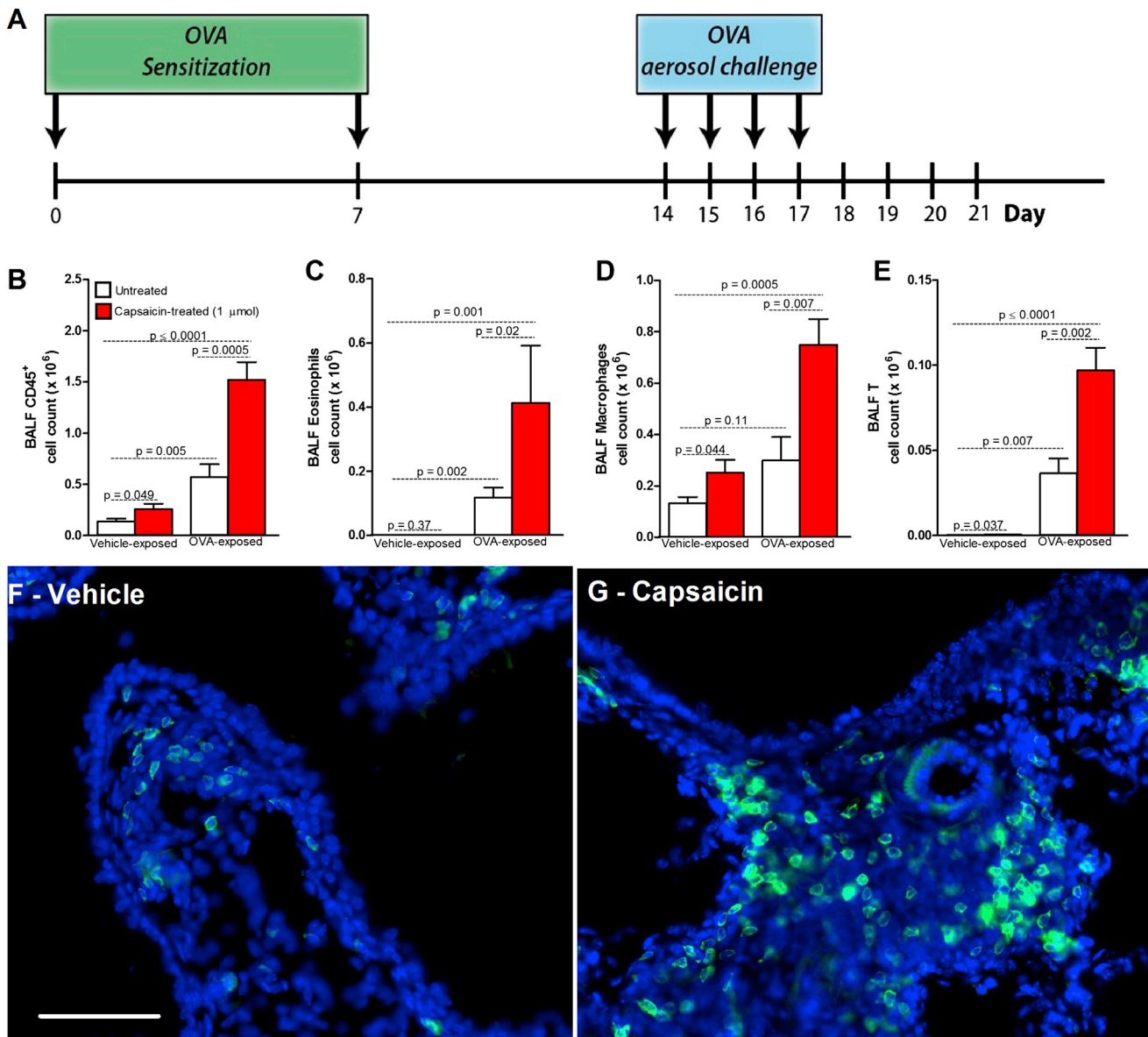
increased the already elevated levels of CD45<sup>+</sup> cells (Figure 1B). These included eosinophils (Figure 1C), macrophages (Figure 1D), and lymphocytes (Figure 1E). The pro-inflammatory effect of capsaicin also was observed in lungs of lymphocyte reporter mice (LCK<sup>+/-</sup>eGFP<sup>+/-</sup>; Figures 1F and 1G). We conclude that exogenous activation of lung nociceptors increases airway leukocytes. The larger effect in OVA-challenged mice than naive mice implies that factors released by nociceptors in inflamed lung augment immune cell recruitment.

### Nociceptor Ablation and Allergic Airway Inflammation

We next used targeted diphtheria toxin-based cell ablation (Palmiter et al., 1987) to explore if nociceptor neurons are required for lung inflammation in the OVA mouse model. Na<sub>v</sub>1.8 is a TTX-resistant voltage-gated sodium channel expressed by nociceptors, including those in the nodose ganglion (Muroi and Undem, 2014) and the majority of nodose ganglion lung afferents (Kwong et al., 2008). Na<sub>v</sub>1.8-Cre<sup>+/-</sup>DTA<sup>+/-</sup> mice are devoid of all Na<sub>v</sub>1.8-expressing nociceptors (Stirling et al., 2005) and have no response to noxious heat, mechanical stimuli, or capsaicin (Chiu et al., 2013). In WT mice and Na<sub>v</sub>1.8-Cre<sup>+/-</sup>DTA<sup>+/-</sup> littermate control mice (with intact nociceptors), numbers of CD45<sup>+</sup> cells, including eosinophils and lymphocytes, increased substantially in BALFs obtained on protocol day 21 (4 days after the last inhaled OVA challenge) (Figures 2A–2D). In contrast, OVA-exposed Na<sub>v</sub>1.8-Cre<sup>+/-</sup>DTA<sup>+/-</sup> mice (with ablated nociceptors) showed significantly dampened immune cell infiltration on day 21, with fewer eosinophils, macrophages, and lymphocytes (Figures 2A–2D).

### Silencing Airway Nociceptors in Non-inflamed Lungs

Since ablating nociceptors during development might conceivably cause compensatory changes in the lung and immune system, we shifted to a strategy to temporarily silence afferents in the adult lung using large-pore ion channels as a drug entry port for charged sodium channel blockers to produce targeted action potential blockade (Binshtok et al., 2007; Brenneis et al., 2013). We first assessed the efficiency of sensory neuron silencing using QX-314, a permanently charged quaternary derivative of lidocaine, in reversing neuropeptide release from nociceptors and consequent vascular leakage in the lung. Activating lung nociceptors in naive mice with capsaicin (1 μmol, 20 μl instilled intranasally) induced significant increases in CGRP levels in BALFs collected 60 min later (Figures 3A–3D). Pre-treatment with QX-314 (100 μM) by inhaled nebulizer 1 hr prior to the capsaicin (when no large-pore channels were activated) failed to alter subsequent capsaicin-induced increases in the neuropeptide, showing that, when administered by itself in the non-inflamed lung, QX-314 fails to block nociceptors, an expected result since there is no portal for entry into nociceptors and it is ineffective when administered extracellularly (Binshtok et al., 2007; Figure 3A). However, when administered just after capsaicin administration, which opens TRPV1 channels, QX-314 blocked CGRP release into BALF (Figure 3B). Co-administration of QX-314 with capsaicin also blocked development of plasma extravasation in the lung, a measure of capillary permeability due to neuropeptide release (Figure 3C). These data show that, when large-pore channels are exogenously activated by TRP



**Figure 1. Nociceptor Activation Promotes Airway Inflammation**

(A) Inflammation induced in mice following i.p. allergen (OVA) sensitization (days 0 and 7, with AIOH) and inhaled challenge (days 14–17). Drugs/vehicle was administered at peak of OVA-induced inflammation (day 18) and impact was assessed on day 21.

(B–E) Capsaicin (B–E; 1 μmol, red bar) or vehicle (white bar) instilled (intranasally) on day 17 after vehicle or OVA challenge (24 hr prior to BALF measurement). Capsaicin increased airway CD45<sup>+</sup> (total immune) (B), eosinophil (C), macrophage (D), and lymphocyte (E) cell counts.

(F and G) T cell influx (green, LCK-Cre<sup>+/+</sup>-eGFP<sup>+/+</sup>) in control lungs following vehicle (F) or capsaicin (G; 1 μmol) instillation is shown. Scale bar, 10 μm. Mean ± SEM; two-tailed unpaired Student's t test (n = 6–10 animals/group, two cohorts).

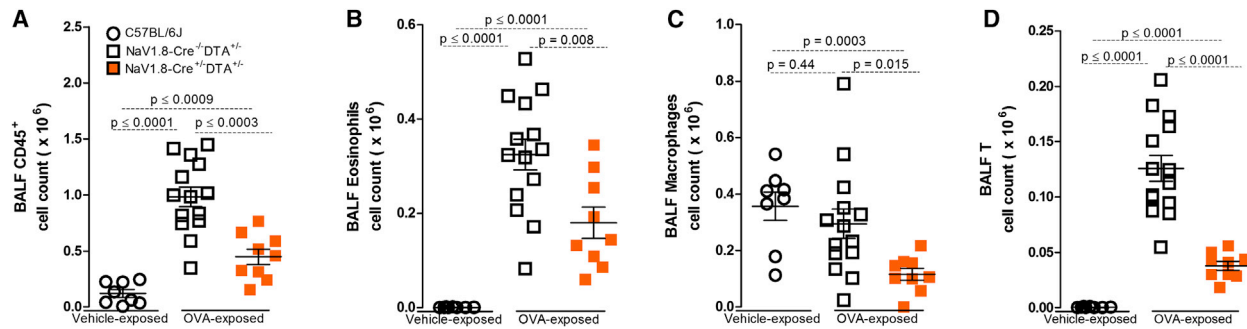
channel agonists, QX-314 can enter into lung nociceptors and inhibit them.

#### Silencing Airway Nociceptors in Inflamed Lungs

Reduced OVA-induced allergic airway inflammation in TRPA1-KO mice (Caceres et al., 2009) and following TRPV1-small interfering RNA (siRNA) knockdown (Mabalirajan et al., 2013; Rehman et al., 2013) indicates activation of these channels by endogenous ligands during allergic inflammation, providing the

opportunity to target QX-314 into nociceptors in inflamed lungs without the need for administration of an exogenous TRP channel agonist. When QX-314 was administered 60 min prior to a capsaicin challenge to OVA-challenged mice on day 18, at the height of inflammation, capsaicin-evoked neuropeptide release was, unlike the situation in naive mice, markedly decreased (Figure 3D), showing that QX-314 treatment by itself in OVA-exposed mice locally blocked nociceptors. To further assess afferent silencing, intranasal capsaicin (500 nmol) was instilled





**Figure 2. Lung Sensory Neuron Ablation Reduces Airway Allergic Inflammation**

(A–D) Four days after last aerosol challenge (day 21), OVA-exposed mice developed CD45<sup>+</sup> cell count increases in BALF (A), including eosinophils (B) and lymphocytes (D), but not macrophages (C). Ablation of NaV1.8- (NaV1.8<sup>+/−</sup>DTA<sup>+/−</sup>, orange squares; A–D) expressing sensory neurons significantly decreased these levels. Mean ± SEM; two-tailed unpaired Student's *t* test (*n* = 4–12 animals/group, one to two cohorts).

in OVA-challenged mice, and nodose ganglion neuron activation was measured by phospho-ERK (p-ERK) immuno-labeling. When OVA-challenged mice were pre-exposed to QX-314 (100 μM) 1 day prior to p-ERK measurements, fewer lung afferent neurons, identified by retrograde Dil-label (Figure S1), were p-ERK positive than in vehicle-treated mice (18.5% versus 86.5% [Figures S1A–S1D]), demonstrating that inhaled QX-314 silences lung nociceptors in OVA-treated mice. Activation of lung nociceptor TRP channels, either by capsaicin in naive animals or by endogenous ligands during allergic inflammation, permits sufficient QX-314 entry to block local nociceptor activity (Figures S1E–S1G).

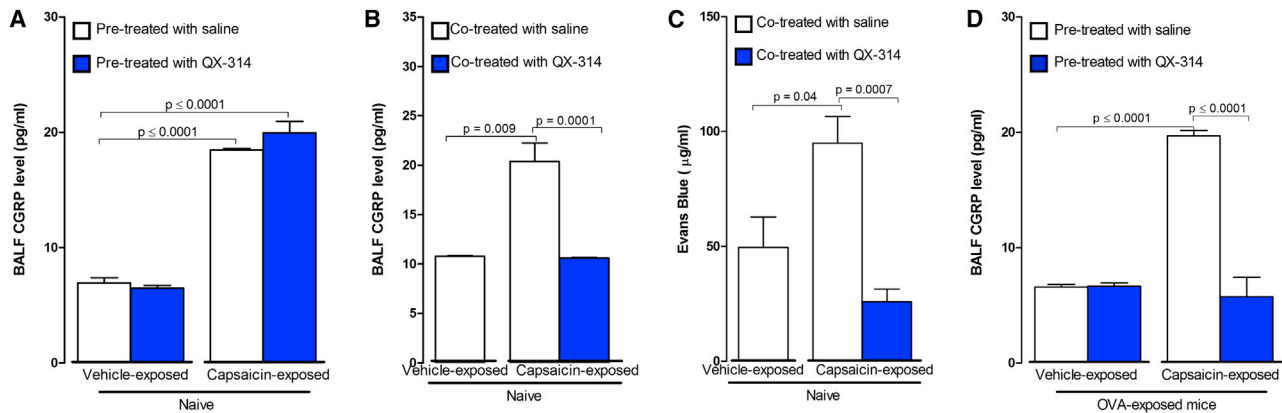
### Nociceptor Silencing and Allergic Airway Inflammation

Single 100-μM treatment with QX-314 on day 18, after OVA inhalation challenges on days 14–17 in OVA-sensitized mice, substantially reduced the immune cell infiltration normally seen on day 21, with decreased BALF CD45<sup>+</sup> cells (total immune cells) and decreased eosinophils, macrophages, and lymphocytes (Figures 4A–4D). Similar results were seen in H&E-stained lung tissue (Figures 4E–4G). The QX-314 effects were independent of sex, age (8 versus 16 weeks), and strain (C57BL/6 versus BALB/c) (data not shown). In addition, this treatment decreased by 1.75-fold the airway basement membrane thickening (Figure 4H). We also tested if sensory neuron silencing reversed type 2 inflammation in the clinically relevant HDM model (Johnson et al., 2004). Briefly, 8-week-old male BALB/c mice were sensitized with HDM (days 1–5) and challenged on days 8–10. On day 15, HDM-challenged mice showed increased BALF CD45<sup>+</sup> cells (Figure 4I) and lymphocytes (Figure 4J), both of which were reduced by QX-314 (100 μM, day 12) treatment. To establish if this was general to all types of allergic inflammation, we analyzed the impact of silencing in a type 1-skewed allergic airway inflammation model. Here, instead of sensitizing mice with aluminum hydroxide and OVA as in all the experiments above, which produces a Th2-skewed inflammation (Bogaert et al., 2011), mice were sensitized with a mixture of complete Freund's adjuvant (CFA) and OVA (Bogaert et al., 2011), which models non-eosinophilic Th1-skewed asthma. These mice had a ~2-fold increase in the Th1/Th2 ratio (defined as CD4<sup>+</sup>IFNγ<sup>+</sup> cells/CD4<sup>+</sup>IL4<sup>+</sup> cells) compared to AIOH/OVA-sensitized mice

(Figure 4K). QX-314 failed to impact levels of CD45<sup>+</sup> cells (Figure 4L), eosinophils, macrophages, and lymphocytes on day 21 in this model (data not shown). Sensory neuron effects on inflammation are context dependent and contribute to type 2 models of allergic airway inflammation, but not a type 1 model.

Inhaled lidocaine, a short-acting sodium channel blocker, has anti-inflammatory effects on murine asthma models (Serra et al., 2012) and in patients (Weiss and Patwardhan, 1977), but directly suppresses immune cells (Serra et al., 2012). We therefore investigated if QX-314 has direct effects on immune cells. QX-314 had no impact on in vitro survival, chemotaxis, and activation of lung macrophages and eosinophils (data not shown), and exposure to QX-314 (up to 1 mM) had no effect on Th0, Th1, and Th2 cell proliferation or cytokine production in vitro (data not shown). We conclude that the immune-suppressing actions of QX-314 are due to nociceptor silencing. The anti-inflammatory effect of nociceptor silencing with QX-314 was replicated with a chemically distinct sodium channel blocker, a permanently charged derivative of mexiletine, N-methyl-mexiletine (NMM). When NMM was administered by nebulizer (on day 18) to OVA-treated mice, BALF-harvested immune cell counts were significantly reduced (Figure S2), albeit with less potency than QX-314. The same dose of uncharged mexiletine failed to resolve the OVA-mediated allergic airway inflammation (Figure S2). Thus, the ability of charged compounds to penetrate into nociceptors through activated large-pore ion channels enables the blockade of sodium channels for longer periods, and this is critical to how silencing sensory neurons reduces inflammation.

We next examined the effect of sensory neuron silencing on bronchial hyperresponsiveness. Both genetic ablation of lung afferent neurons (data not shown) and QX-314 silencing on day 18 reversed OVA-mediated bronchial hyperresponsiveness, as measured on day 21 by ED<sub>200</sub>R<sub>L</sub> (Figure 4M), lung elastance (Figure 4N), and resistance (Figure 4O). Specifically, the dose of methacholine (MCh) required to produce a 200% increase in R<sub>L</sub> (i.e., log ED<sub>200</sub>R<sub>L</sub>) decreased significantly (*p* < 0.001) from 6.38 ± 0.74 in control mice to 0.79 ± 0.06 after OVA challenge, while QX-314 increased by ~3-fold the log ED<sub>200</sub>R<sub>L</sub> values in OVA-challenged mice (Figure 4M). NMM also reduced airway hyperresponsiveness (data not shown).



**Figure 3. Airway Sensory Neuron Silencing with QX-314 Abolishes Capsaicin-Induced Peptide Release and Vascular Leak**

(A and B) To assess nociceptor-mediated peptide release, capsaicin (1  $\mu$ mol, intranasally) was administered to naive mice. BALF CGRP levels were increased 1 hr following the capsaicin challenge (A and B). QX-314 (100  $\mu$ M) treatment 1 hr prior to the capsaicin did not alter capsaicin-induced CGRP levels (A), but QX-314 (100  $\mu$ M), when administered immediately after the capsaicin, blocked the CGRP rise (B) ( $n = 4$ –15 animals/group, two to three cohorts). (C and D) Reduction in vascular leak (C) following co-administration of capsaicin (1  $\mu$ mol) with QX-314 (100  $\mu$ M) ( $n = 4$ –5 animals/group, one cohort). BALF CGRP levels increased following capsaicin (1  $\mu$ mol) instillation in OVA-challenged mice (D), and this was blocked by QX-314 pre-treatment 1 hr before (100  $\mu$ M). Mean  $\pm$  SEM; two-tailed unpaired Student's  $t$  test ( $n = 5$ –12 animals/group, two cohorts).

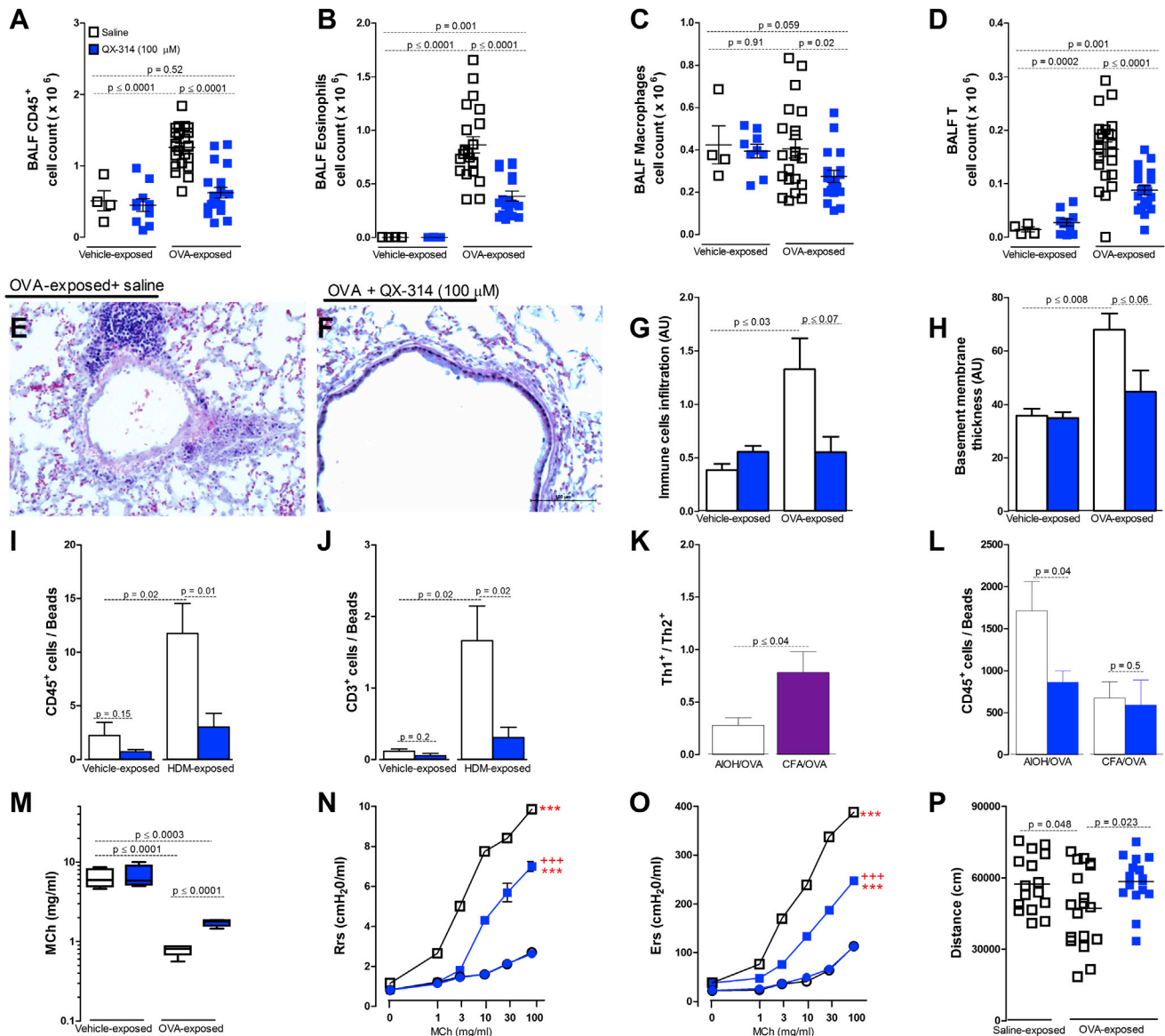
To functionally explore the consequences of airway hyperresponsiveness in mice, we measured daily voluntary wheel running. Individual animals were followed over the course of the allergen protocol (Figure 1A) and their daily level of voluntary exercise (1 hr, dark, 21:00–1:00 hr) was analyzed. At the peak of inflammation (day 18), mice showed a significant decrease in travel distance  $\sim 25\%$  compared to their pre-challenge period (day 13) (Figure 4P), and this was matched by an equivalent decrease in exercise duration. Voluntary wheel running distance was rescued by QX-314 treatment, measured 24 hr later (Figure 4P). Local silencing of lung nociceptors by a single exposure to a charged sodium channel blocker at the peak of allergen-induced inflammation reduced two cardinal features of allergic asthma: immune infiltration and bronchial hyperresponsiveness.

### IL-5 Activates Lung Nociceptors

Our data suggest that nociceptors are activated during exposure to allergens in sensitized animals, but the mechanisms responsible are unknown. Skin nociceptors are activated by the cytokine IL-1 $\beta$  (Binshtok et al., 2008), and we hypothesized that similar signaling could occur during allergic airway inflammation. To look for candidates, we asked if silencing lung nociceptors with QX-314 reduced BALF levels of cytokines, and we discovered decreased IL-5 (Figure 5A), eotaxin-2, IL-4, IL-9, IL-10, IL-13, IP-10, MCP-1, TNF- $\alpha$ , and TARC, but not IL-17, IL-23, IL-27, IL-28 $\beta$ , INF- $\gamma$ , and RANTES (Table S1). Transcript profiling of the naive nodose ganglion revealed that lung Na $_v$ 1.8 $^+$  nociceptors express receptors for some of these cytokines, including IL-33R, INF- $\gamma$ -R, IL-4R, IL-9R, and IL-5R (Figure 5B). We therefore explored if eotaxin-2, IL-13, and IL-5 could activate cultured nodose neurons, using calcium imaging of neurons from control, OVA-sensitized (day 13) and OVA-sensitized and -challenged (day 21) mice, and we observed that the latter condition produced maximal responses (data not shown) so we chose

to perform subsequent experiments on neurons from OVA-challenged mice. Exposing neurons to eotaxin-2 or IL-13 at concentrations up to 10  $\mu$ g/ml produced small concentration-dependent calcium increases in a few neurons (for IL-13, 3/469 KCL $^+$  cells, 0.6%; and eotaxin-2, 26/403 KCL $^+$  cells, 6.45%). However, larger responses in more neurons were observed for IL-5 (150/788 KCL $^+$  cells, 19.04%) (Figure 5C).

IL-5, along with IL-13, is a major effector cytokine in asthma, implicated in airway inflammation, hyperresponsiveness, and remodeling, as well as mucus metaplasia (Ortega et al., 2014). To characterize nodose neurons that responded to IL-5, we exposed them to different TRP channel ligands and found that 55% (42/78) of IL-5-responsive cells also responded to capsaicin and 41% (32/78) to AITC (Figure 5D), while 55% of capsaicin- and 100% of AITC-responsive cells were IL-5 responsive (data not shown). IL-5-responsive nodose ganglion neurons were predominantly of small and medium sizes (Figure 5E). Only 1.3% of cultured nodose ganglion from Na $_v$ 1.8-ablated mice responded to IL-5 in comparison to  $\sim 25\%$  in littermate controls (Figure 5F). Removing extracellular calcium eliminated the IL-5 signal (Figure 5G), suggesting flux through membrane-bound channels. Whole-cell patch clamp recordings in current-clamp mode from small cells (capacitance  $<45$  pF) that had responded to IL-5 by calcium imaging showed that IL-5 perfusion (1  $\mu$ g/ml) depolarized the cells by  $\sim 6$  mV (Figures 5H and 5I;  $n = 12$  cells), an effect that was reversible after extensive washout (more than 20 min). Before IL-5 perfusion, cells had a stable resting membrane potential (RMP) with no spontaneous action potential firing; however, occasional spontaneous spiking was observed following IL-5 treatment (2/12 patched cells). The current needed to trigger an action potential (rheobase) was smaller when IL-5 was present (Figures 5J and 5K;  $n = 7$  cells), but no difference in spiking frequency during a ramp current was observed (data not shown). We conclude that nociceptors can be directly activated by IL-5.



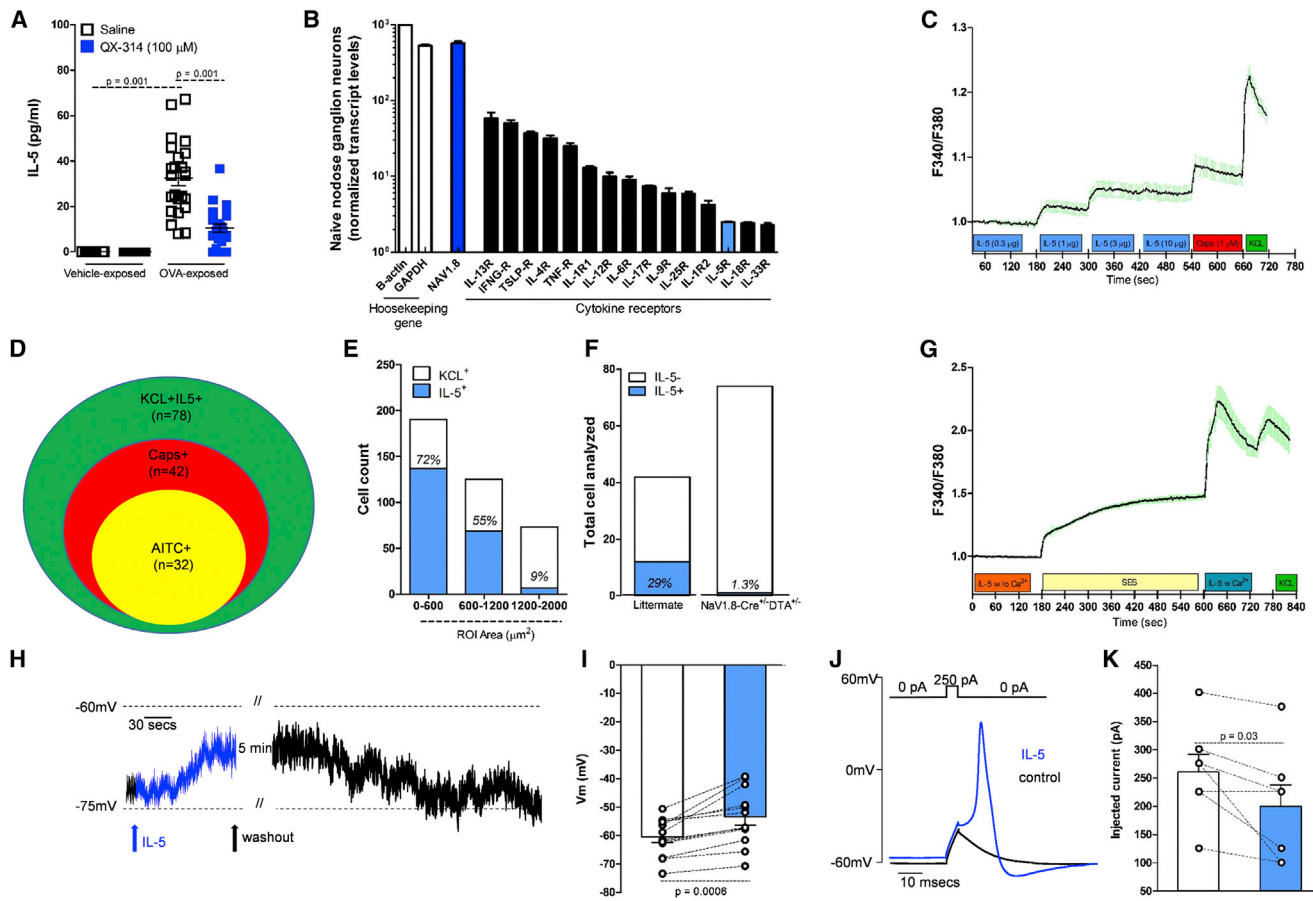
**Figure 4. Airway Sensory Neuron Silencing Reduces Lung Inflammation and Hyperresponsiveness**

(A–D) OVA-exposed mice develop increased CD45<sup>+</sup> immune cell counts in BALF (A), which include eosinophils (B) and lymphocytes (D), but not macrophages (C). (E and F) Silencing lung sensory neurons with QX-314 (100  $\mu$ M, 72 hr prior to measurement; blue squares) decreased these immune cell responses (A–H). Representative H&E-stained sections of OVA-exposed lungs treated with saline (E) or QX-314 (100  $\mu$ M; F) are shown. Scale, 100  $\mu$ m. (G and H) Immune cell infiltration severity (G) and basement membrane thickening (H) measured by tissue morphometry in HE sections are shown. (I and J) HDM challenge increased BALF CD45<sup>+</sup> (I) and lymphocyte (J) counts, an effect reversed by QX-314 (100  $\mu$ M). (K and L) CFA/OVA sensitization produced a greater increase in Th1/Th2 cell ratio than AIOH/OVA sensitization (K). QX-314 (100  $\mu$ M) reduced BALF CD45<sup>+</sup> cell counts in the AIOH/OVA-, but not the CFA/OVA-sensitized model (L). Mean  $\pm$  SEM; two-tailed unpaired Student's t test ( $n = 4$ –25 animals/group, four cohorts). (M–P) Change in airway reactivity measured as ED<sub>200</sub>R<sub>L</sub> (M), resistance (N), and elastance (O) in OVA-exposed mice treated with QX-314 (D–F; 100  $\mu$ M, day 18) or saline; difference in vehicle- (\*\*\*) and OVA-exposed (\*\*\*\*) groups,  $p < 0.001$ . OVA-exposed mice performed less voluntary wheel running (P) (1-hr assessment) than control mice, an effect reversed by QX-314 (100  $\mu$ M, day 18) assessed 24 hr after silencing. Two-tailed unpaired Student's t test ( $n = 4$ –40 animals/group, two to three cohorts).

### Lung Nociceptors Activate ILC2 and Lymphocytes

Lung epithelial and dendritic cells are activated upon an allergen challenge to stimulate resident lung ILC2 cells (Licona-Limón et al., 2013). ILC2 cells are implicated in the onset of asthma, and they are directly activated by IL-25 and IL-33 released

from goblet and dendritic cells to act upstream of Th2 cells, B cells, eosinophils, smooth muscle cells, and macrophages. ILC2 cells produce IL-5, IL-9, and IL-13 and are resident in the airways, where they orchestrate type 2 immunity (Barnig et al., 2013; Licona-Limón et al., 2013). Activation of ILC2 cells typically



**Figure 5. IL-5 Activates  $\text{Nav1.8}^+$  Nodose Ganglion Neurons**

(A) IL-5 levels in BALFs increased in OVA-challenged mice, an effect reversed by QX-314 (100  $\mu\text{M}$ ). (B) Transcript profiling of naive nodose ganglia reveals basal expression of cytokine receptors including IL-5R. (C) Cultured nodose ganglion neurons from OVA-exposed mice show a dose-dependent calcium increase in response to IL-5 (0.3–10  $\mu\text{g/ml}$ ). (D) Venn diagram shows overlapping populations of  $\text{KCL}^+\text{IL-5}^+$  and AITC- or capsaicin-responsive neurons. (E and F) IL-5-responsive cells are mainly small and medium sized (E) and nearly null in OVA-exposed  $\text{Nav1.8-Cre}^{+/-}\text{DTA}^{+/-}$  mice (F). (G) IL-5-mediated calcium increase is absent in 0 external  $\text{Ca}^{2+}$ . (H and I) Current-clamp recordings of small cells (capacitance <45 pF) show that IL-5 (1  $\mu\text{g/ml}$ ) depolarizes RMP by  $\sim 6$  mV ( $n = 12$  cells). (J and K) The current needed to trigger an action potential (rheobase) is smaller when IL-5 is present ( $n = 7$  cells). Mean  $\pm$  SEM; two-tailed unpaired (A–G) and paired (H–K) Student's  $t$  test ( $n = 3$  biological replicates per group).

precedes that of Th2 cells, although transactivation and cooperation occur (Halim et al., 2014; Licona-Limón et al., 2013). Typically activated ILC2 cells produce IL-13 and have higher levels of the IL-33R, ST2, while a subset of Th2 cells produces IL-5 and IL-13, the predominant effector cytokines in asthma. We therefore set out to assess if nociceptor ablation had any effect on ILC2 cell or  $\text{CD4}^+$  cell activation, as measured by surface markers and cytokine production. To do this, we examined early immune changes after two OVA challenges on day 16. Relative to littermate controls ( $\text{Nav1.8-Cre}^{+/-}\text{DTA}^{+/-}$ ), OVA-exposed mice with ablated nociceptors ( $\text{Nav1.8-Cre}^{+/-}\text{DTA}^{+/-}$ ) showed decreased BALF levels of  $\text{CD45}^+$ , as seen previously (Figures 2A and 6A). In addition, there were fewer  $\text{CD4}^+$  (Figure 6B) and ILC2 (Figure 6C) cells. Moreover, nociceptor-ablated mice demonstrated decreased numbers of IL-13 $^+$  ILC2 (Figure 6D) and lower expression of CD25 (Figure 6E) and ST2 (Figure 6F), ILC2 activation

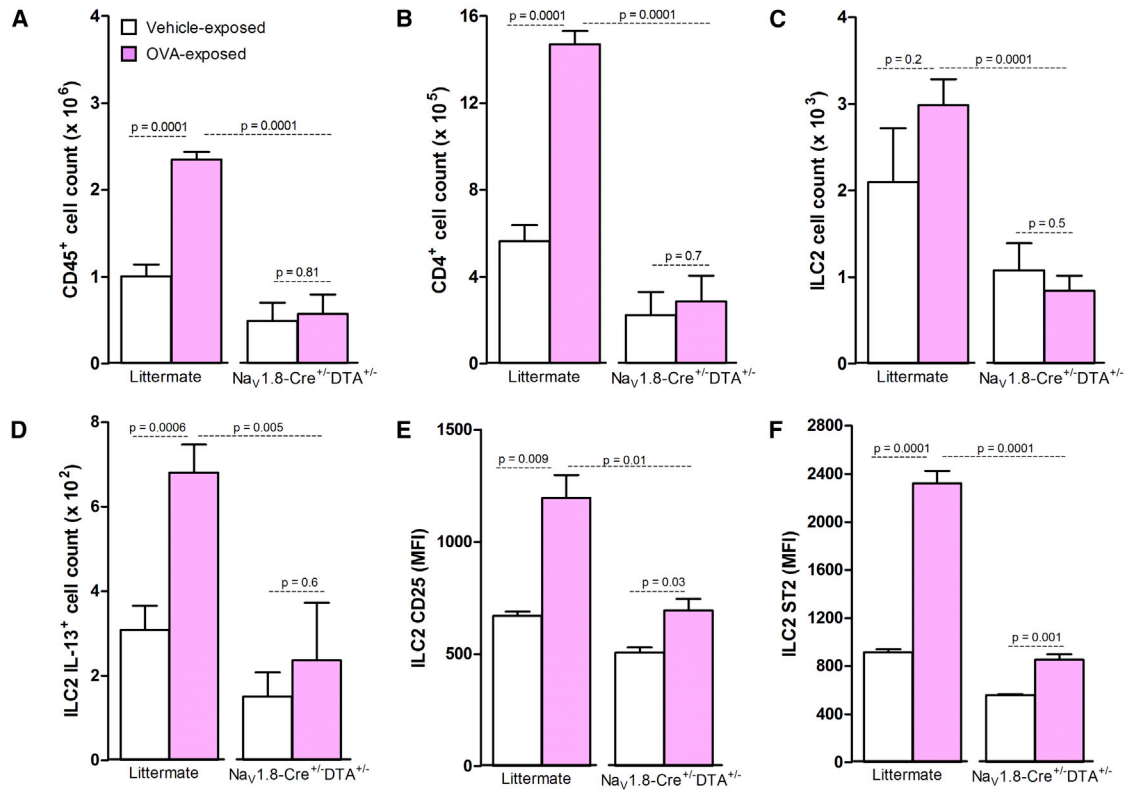
markers (Wilhelm et al., 2011). Similarly, QX-314 administration at the peak of inflammation (100  $\mu\text{M}$ , day 18) reduced the rise in total  $\text{CD4}^+$ ,  $\text{CD4}^+\text{IL-5}^+$ , and  $\text{CD4}^+\text{IL-13}^+$  cells (BALF, on day 21). Thus, both genetic ablation and pharmacologic silencing of nociceptors result in decreased activation of ILC2s and Th2 effector cytokine production.

### Lung Nociceptors Release VIP

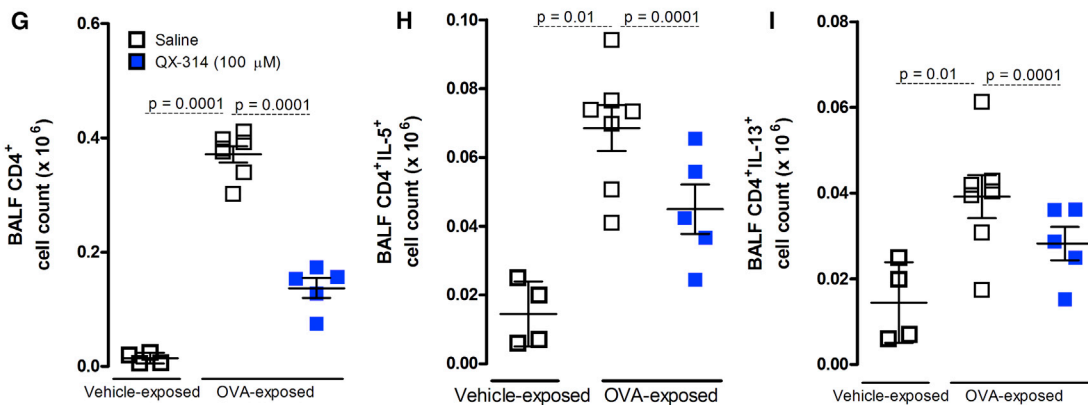
The effect of sensory neurons on ILC2 and  $\text{CD4}^+$  cells likely involves multiple pathways and several candidate neuropeptides could be involved. Genomic analysis of lung-resident ILC2 cells has revealed expression of receptors for several neuropeptides released by sensory neurons, including SP, CGRP, and VIP (Saenz et al., 2013), providing an opportunity for nociceptors to directly communicate with these cells. We chose to examine VIP, both because it is present at high levels in nodose ganglion



## Early stage - day 16



## Late stage - day 21

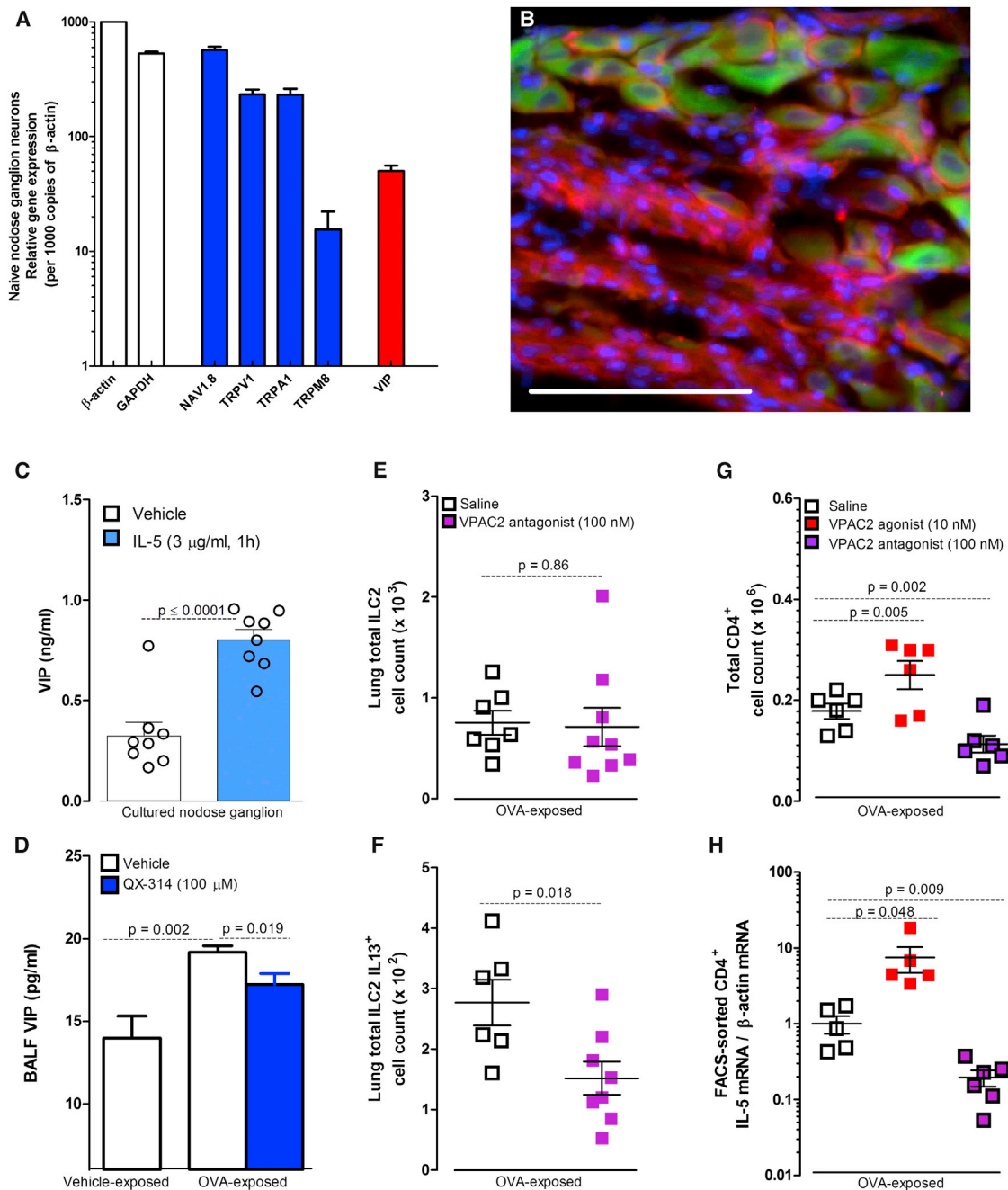


**Figure 6. Sensory Neurons Control ILC2 and CD4<sup>+</sup> Cell Recruitment and Activation in the Lung**

(A–F) On day 16, OVA-exposed mice (pink bars) with intact nociceptors (littermate;  $Na_v1.8^{-/-}DTA^{+/+}$ ) had higher whole-lung CD45<sup>+</sup> (A), CD4<sup>+</sup> (B), ILC2 (C), ILC2 IL-13<sup>+</sup> cell counts (D), as well as ILC2 CD25 (E) and ST2 mean fluorescence intensities (MFIs) (F) than mice with ablated nociceptors ( $Na_v1.8^{+/+}DTA^{-/-}$ ). (G–I) QX-314 treatment (100  $\mu$ M, day 18; blue squares) decreased OVA-induced CD4<sup>+</sup> cells on day 21 (G), including CD4<sup>+</sup>IL5<sup>+</sup> (H) and CD4<sup>+</sup>IL13<sup>+</sup> (I) cells, relative to control mice (white squares). Mean  $\pm$  SEM; two-tailed unpaired Student's *t* test (*n* = 4–9 animals/group, one to two cohorts).

neurons (Figure 7A) and because its two G protein-coupled receptors VPAC1 and VPAC2 are expressed by and exert a powerful regulatory effect on T cells, including differentiation, migration, and cytokine production (Delgado et al., 2000). We found that  $Na_v1.8^{+}$  nodose ganglion neurons express VIP (Fig-

ure 7B), including lung afferents in OVA-exposed mice (Dil retrogradely labeled) (Figure S3A). Cultured nodose ganglion neurons stimulated with capsaicin (Figure S3B) or IL-5 (Figure 7C) released VIP, while BALF from OVA-exposed mice contained elevated VIP compared to vehicle-challenged mice and this



**Figure 7. IL-5 Provokes Sensory Neuron Release of VIP to Activate ILC2 and Recruit CD4<sup>+</sup> Cells**

(A) Gene profiling of naive Nav1.8<sup>+</sup> nodose ganglion neurons reveals expression of nociceptor markers and VIP.

(B) A VIP reporter mouse (VIP-Cre<sup>+/+</sup>-EGFP/td-tomato<sup>+/+</sup>) reveals that ~80% of Nav1.8<sup>+</sup> (magenta) nodose ganglion neurons express VIP (green) (scale bar, 50  $\mu$ m).

(C) IL-5 (1 hr, 3  $\mu$ g/ml) induced VIP release from nodose ganglion neurons from OVA-exposed mice.

(D) These mice have elevated BALF VIP levels on day 21 compared to vehicle-exposed mice (white bar), which is decreased by QX-314 treatment (100  $\mu$ M, day 18; blue bar).

(E and F) In OVA-exposed lungs, the VPAC2 antagonist PG 99465 (100 nM, every 12 for 48 hr starting on day 16; pink squares) did not impact resident ILC2 numbers (E) but reduced ILC2 activation IL-13<sup>+</sup> cells (F).

(G and H) The VPAC2 agonist BAY 55-9837 (10 nM, every 12 for 96 hr; red squares) increased and the VPAC2 antagonist PG 99465 (100 nM, every 12 for 96 hr; pink squares) decreased CD4<sup>+</sup> cell numbers (G) and IL-5 mRNA expression in FACS-sorted CD4<sup>+</sup> cells (H). Mean  $\pm$  SEM; two-tailed unpaired Student's *t* test (*n* = 3–14 animals/group, one to two cohorts).

was reduced by inhaled QX-314 (Figure 7D). Nodose ganglions from OVA-exposed mice expressed more VIP transcripts than naive mice (data not shown). These data indicate that VIP is released in the inflamed lung and this can be blocked by silencing neurons with a charged sodium channel blocker.

### VIP Acts on ILC2 and Th2 Cells

We next asked if VIP can act on ILC2 and T cells. We isolated CD4<sup>+</sup> T cells from spleens and lymph nodes of naive mice, and we differentiated them *in vitro* under Th1-, Th2-, and Th17-skewing conditions, followed by exposure to VIP. When CD4<sup>+</sup> T cells cultured under Th2-skewing conditions were exposed to recombinant mouse VIP (500 nM), their transcript levels of IL-13 (data not shown) and IL-5 (Figure S3C) increased, suggesting that VIP contributes to the competence of Th2 cells to transcribe these type 2 regulatory cytokines. VIP (0–1.0  $\mu$ M) did not affect transcription of IL-5 or IL-13 in T cells cultured under Th0-, Th1-, and Th17-skewing conditions (data not shown). The VPAC2 antagonist PG 99465 administered early in the OVA challenge (100 nM in 50  $\mu$ l, intranasally, every 12 for 48 hr starting on day 14) significantly decreased CD4<sup>+</sup> cells in the whole lung on day 16, particularly those producing IL-13 (Figures S3D and S3E). Resident lung ILC2 numbers were not affected by VPAC2 blockade (Figure 7E), but their activation was significantly reduced, as indicated by decreased IL-13 intracellular staining (Figure 7F) and decreased surface expression of ST2 (Figure S3G). Impaired ILC2 activation may be important given the key role for this population in the initiation of allergic airway inflammation (Halim et al., 2014). When the VPAC2 antagonist was given for a longer period, over the full duration of OVA challenge (twice daily on days 14–18), it reduced BALF total immune (CD45<sup>+</sup>) (Figure S3F) and CD4<sup>+</sup> cell numbers (Figure 7G), as well as IL-5 mRNA expression in CD4<sup>+</sup> T cells sorted via fluorescence-activated cell sorting (FACS) from BALF on day 19 (Figure 7H). In contrast, a VPAC1 antagonist did not alter immune cell number or activation (data not shown). Activation of VPAC2 in OVA-exposed mice with a selective agonist further increased CD4<sup>+</sup> cell numbers (Figures 7G, S3H, and S3I) and IL-5 mRNA levels (Figure 7H). VIP signaling through VPAC2 may play, therefore, a significant role in the activation of ILC2 cells and cytokine expression by CD4<sup>+</sup> T cells, and may account, at least partially, for the ability of nociceptors to amplify type 2 inflammation in the lung.

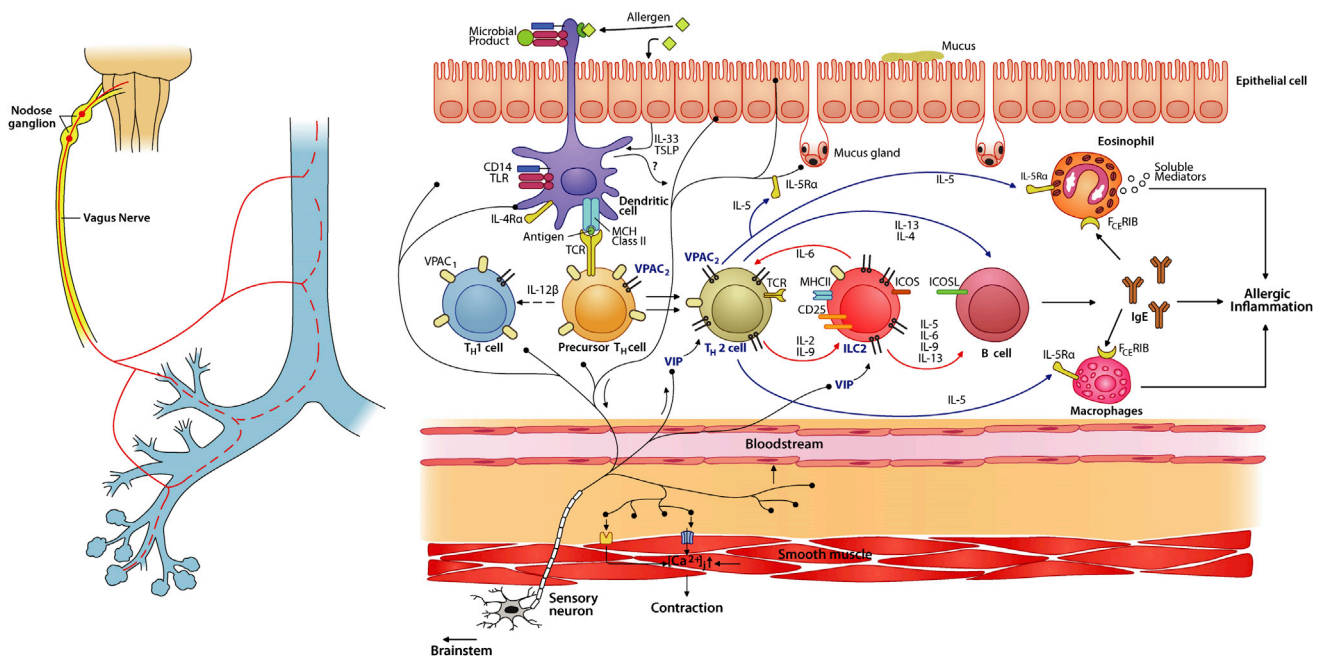
### DISCUSSION

The nervous and immune systems work in concert to protect against environmental dangers and also can fail together in disease states, including asthma. Nociceptor-immune interactions appear to contribute to arthritis-induced inflammation (Levine et al., 1984), colitis (Engel et al., 2011), psoriasis (Riol-Blanco et al., 2014), and acute lung injury (Su et al., 2005); however, the mechanisms by which this happens remain uncertain. Here we have demonstrated a complex interplay between nociceptors and immune cells. Through pharmacological silencing and genetic ablation of nociceptors in the OVA- and HDM-induced mouse models of airway inflammation, we have uncovered a major upstream contribution of these fibers to the

generation and persistence of type 2 allergic airway inflammation and bronchial hyperresponsiveness. Whereas direct stimulation of lung nociceptors with capsaicin promotes neuropeptide release and immune cell infiltration, silencing sensory neurons or ablation of Na<sub>v</sub>1.8<sup>+</sup> nociceptors substantially reduces immune cell infiltration and bronchial hyperresponsiveness. We also have identified a means of communication between sensory neurons and the select set of immune cells pivotal for the initiation and amplification of type 2 allergic inflammation. We found that IL-5, a type 2 effector cytokine produced by multiple immune cells, directly activates sensory neurons leading to secretion of the neuropeptide VIP, and that VIP acts on ILC2 and CD4<sup>+</sup> cells to induce cytokine production. In addition, we discovered that charged sodium channel blockers, which specifically and locally silence TRP-activated sensory neurons, interrupt this signaling loop and reduce well-established inflammation.

Several studies have suggested that nociceptors play a role in adaptive immune responses. A reduction in allergic airway inflammation was shown in TRPA1-KO mice (Caceres et al., 2009) and upon TRPV1-siRNA knockdown (Mabalirajan et al., 2013; Rehman et al., 2013), while C-fiber denervation in rats (produced by neonatal high-dose capsaicin exposure) decreased OX-6<sup>+</sup> dendritic cell numbers in the lung; pulmonary lymphoid infiltration after allergen challenge (hen egg lysozyme) (Kradin et al., 1997); and PAR2-mediated airway constriction, pulmonary inflammation, and edema (Su et al., 2005). Collectively, these findings, together with studies on atopic dermatitis and psoriasis (Riol-Blanco et al., 2014; Wilson et al., 2013), indicate that nociceptors can potentiate adaptive immune responses; however, the mechanisms responsible were not identified in these studies. Here we show that nociceptors are crucial to the generation and persistence of type 2 airway inflammation, identify a pharmacological approach for using this insight therapeutically, and identify some specific mechanisms for these neuro-immune interactions.

Allergic airway hyperresponsiveness may result both from local signaling by immune cells directly to airway smooth muscle cells and through nociceptor-mediated stimulation of sub-epithelial parasympathetic efferents through central and local reflexes. A recent study found that ablation of a subset of TRPV1<sup>+</sup> sensory neurons reduced bronchial hyperresponsiveness (Tränkle et al., 2014). We replicated this finding using Na<sub>v</sub>1.8 ablation, and we extended it by showing similar effects with the pharmacological silencing of pain fibers, including on daily voluntary exercise, a new functional readout of airway hyperresponsiveness. Tränkle et al. reported that ablation of TRPV1 sensory neurons did not reverse airway inflammation, in contrast to our pharmacological and genetic ablation data that revealed such an effect. Explanations for this discrepancy include different allergy models (intranasal allergen versus nebulization), timing of measurements (24 versus 96 hr), assessment methods (Wright-Giemsa stain versus FACS immunophenotyping), and, most importantly, different targeted neuron populations (TRPV1 versus Na<sub>v</sub>1.8). Na<sub>v</sub>1.8 is expressed by  $\geq 80\%$  of nociceptors (Muroi and Undem, 2014) and the majority of nodose ganglion lung afferents (Kwong et al., 2008), while TRPV1 is expressed by only  $\sim 50\%$  of Na<sub>v</sub>1.8 nociceptors. This



**Figure 8. Model of Nociceptor Involvement in Type 2 Inflammation**

During allergen exposure, lung afferents are activated by dendritic and epithelial cells. Dendritic cells also polarize precursor Th cells into Th2 cells. Activated nociceptors release VIP, which stimulates lung-resident ILC2 and newly differentiated Th2 cells via the VPAC2 receptor. Type 2 cytokines, including IL-5 and IL-13, are released by ILC2 and Th2 cells and initiate the chemotaxis and activation of eosinophils and macrophages, IgE secretion by B cells, mucus production by goblet cells, and smooth muscle contraction, culminating in allergic inflammation and bronchial hyperresponsiveness. IL-5 activates nociceptors to trigger VIP and other neuropeptide release, leading to additional IL-5 production. Scheme inspired by [Licón-Limón et al. \(2013\)](#) and [Vercelli \(2008\)](#).

suggests that a broad population of nociceptors needs to be silenced or ablated to interrupt their pro-inflammatory actions.

Th2 and ILC2 cells are both critical to the pathogenesis of allergic airways disease. We found that after an allergen challenge in Th2-skewed-sensitized mice, sensory neurons stimulate cytokine production in CD4<sup>+</sup> and ILC2 cells. ILC2 cells are implicated in the onset of asthma and are directly activated by IL-25 and IL-33 released from goblet and dendritic cells, and they act upstream of Th2 cells, B cells, eosinophils, smooth muscle cells, and macrophages ([Walker et al., 2013](#)). Therefore, activation of sensory neurons on allergen exposure may be a very early event in igniting inflammation in a sensitized animal. We asked how sensory neurons may communicate with Th2 and ILC2 cells to potentiate/initiate inflammation. Activation of nociceptors, through calcium influx, induces the release of neuropeptide transmitters from vesicles in the peripheral terminals. In the lung, release of these neuropeptides occurs in response to chemical agents (capsaicin, citric acid, nicotine, cigarette smoke, histamine, and bradykinin) and physical factors (mechanical probing, dry gas, and hypertonic aerosols) ([Bessac and Jordt, 2008](#)). Classically, such neuropeptide release (with a focus on substance P and CGRP) was thought to trigger only local increased capillary permeability and vasodilation; but, there have been suggestions that nociceptor-mediated peptide release may contribute to inflammation in arthritis ([Levine et al., 1984](#)), colitis ([Engel et al., 2011](#)), psoriasis ([Riol-Blanco et al., 2014](#)), and acute lung injury ([Su et al., 2005](#)). Lung-resident ILC2 cells express receptors for the sensory neuron neuropep-

tide SP, CGRP, and VIP ([Saenz et al., 2013](#)). Established neuropeptide-mediated immune cell regulation includes the CGRP and VIP biasing of dendritic cells toward the Th2/Th1 response; enhanced dendritic cell migration to the lymph node ([Ding et al., 2008](#)); and activation of mast ([Ansel et al., 1993](#)), Langerhans ([Hosoi et al., 1993](#)), Th2 cells ([Delgado et al., 2000](#); [Ding et al., 2008](#)), and ILC2 cells ([Nussbaum et al., 2013](#)).

Our data suggest that release of VIP by sensory fibers in the lung activates ILC2 and CD4<sup>+</sup> T cells, in addition to their canonical activation by cytokines, and that this contribution is sufficiently large that silencing sensory neurons substantially reduces and increases resolution of allergic inflammation ([Figure 8](#)). The action of VIP on immune cells is context and cell type dependent. Unstimulated Th cells have high basal expression of VPAC1 ([Harmar et al., 1998](#)). T cell antigen receptor stimulation, however, upregulates VPAC2 and diminishes VPAC1 expression, and, in consequence, VPAC2 is the dominant transducer of VIP's effects on Th2 cells ([Lara-Marquez et al., 2001](#)). This shift from VPAC1 to VPAC2 in activated T cells shifts the balance from the delayed-type cellular-protective hypersensitivity responses mediated by Th1 cells to the immediate-type allergic immune responses mediated by Th2 cells. Supporting a role for VPAC2 signaling in type 2 immune responses, transgenic mice with high constitutive VPAC2 expression in T cells show blood eosinophilia, elevated serum IgE levels, greater IgE antibody responses, and cutaneous immediate-type reactions to allergy-producing antigens ([Voice et al., 2001](#)), while VPAC2-KO mice show Th1-associated responses ([Goetzl et al., 2001](#)). Nociceptor



activation and consequent VIP release may elicit, therefore, different types of immune activation, depending on immune cell state. Activation of ILC2 cells by VIP results in IL-5 induction (Nussbaum et al., 2013), something we reproduced here. We further showed that ILC2 activation in the OVA model of airway allergic inflammation is reduced by a VPAC2 antagonist. However, because of the bronchodilator actions of VIP on smooth muscle (Lindén et al., 2003), it is unlikely that VPAC2 antagonists could be used therapeutically for asthma.

What may lead to nociceptor activation after an allergen challenge? Because nodose ganglion neurons express a wide variety of receptors for many cytokines and chemokines upregulated in asthma, we explored the possibility that select mediators could drive nociceptor activation, and we found that IL-5 directly activates nociceptors, leading to calcium influx and release of VIP, which act on immune cells to trigger more IL-5 production. IL-5 is known to be central to asthma-associated pathologies where it promotes eosinophil recruitment and triggers release of eosinophil cationic protein (ECP) and transforming growth factor  $\beta$  (TGF- $\beta$ ) to induce airway smooth muscle inflammation and hyperreactivity (Shi et al., 1998).

We have uncovered a positive feedforward loop, involving IL-5, nociceptors, VIP, ILC2, and Th2 cells, that amplifies type 2 inflammation (Figure 8). There may well be additional mechanisms to activate sensory neurons during such inflammation, for example, thymic stromal lymphopoietin (TSLP), which is implicated in asthma (Gauvreau et al., 2014). TSLP is released from keratinocytes in a mouse model of atopic dermatitis to directly activate a subset of TRPA1<sup>+</sup> sensory neurons (as well as immune cells) to trigger itch (Wilson et al., 2013). Furthermore, in psoriasis-like conditions, skin allergen-sensitized dendritic cells make direct contact with afferent sensory neurons and activate them (Riol-Blanco et al., 2014), although the mediator responsible is not yet identified.

Currently, there is no treatment that accelerates the resolution of inflammation in asthma (Levy et al., 2012). Several drugs control disease symptoms and help abort attacks (Fanta, 2009). Bronchodilators,  $\beta_2$ -agonists, or anti-cholinergics give immediate relief through relaxation of smooth airway muscle (Green et al., 2003). Immune modulators inhibit inflammation, with corticosteroids the primary treatment modality (Green et al., 2003); however, chronic steroid use leads to adverse effects (Locksley, 2010). Despite the effectiveness of current therapy for mild asthma, patients with severe asthma have frequent exacerbations and excess morbidity. Asthma is a heterogeneous condition, but a therapeutic approach that targets nociceptors upstream of type 2 inflammation might provide a broadly effective treatment for many patients (Barnes, 2004). Nevertheless, sensory neuron interaction with immune cells appears to be highly cell and context dependent; for example, during pathogen invasion, the release of neuropeptides (CGRP, galanin, and somatostatin) suppresses innate immune responses (Chiu et al., 2013), in contrast to the amplifying role we report here for type 2 allergic airway inflammation, and treatment would need to be evaluated and individualized accordingly.

In conclusion, the silencing and genetic ablation of nociceptors has uncovered a major upstream contribution of these fibers in the generation and persistence of type 2 allergic airway inflam-

mation and bronchial hyperresponsiveness. Sensory neurons, upon activation by allergen exposure in sensitized animals, control through local neuropeptide release at their peripheral terminals type 2 inflammation, acting on ILC2 and CD4<sup>+</sup> cells. These cells secrete cytokines such as IL-5 that recruit effector immune cells integral to the disease state, and also further activate sensory neurons. Silencing sensory neurons provides, therefore, a potential strategy worth exploring for the treatment of type 2 allergies, potentially including conjunctivitis, rhinitis, and asthma. This strategy is highly specific, only targeting neurons whose TRP channels are activated by inflammation, with expected low systemic side effects (positively charged drugs have limited capability of spreading) and a long duration of action (positively charged compounds should remain trapped inside neurons for prolonged periods), constituting a novel therapeutic approach to allergic airway inflammation centered on interrupting sensory neuron immune signaling.

## EXPERIMENTAL PROCEDURES

All procedures were approved by the Institutional Animal Care and Use Committees of Boston Children's Hospital. Allergic airway inflammation was studied in an OVA-based model (Haworth et al., 2008). On days 0 and 7, mice were sensitized by 200- $\mu$ l i.p. injections of a solution containing 1 mg/ml OVA (Sigma-Aldrich) and 5 mg/ml aluminum hydroxide (Sigma-Aldrich). On days 14–17 (10:00 am), mice were exposed to 6% OVA aerosol for 25 min. QX-314 (100  $\mu$ M) was nebulized on day 18, mice were sacrificed on day 21, and BALF was harvested; cells were isolated, counted, and immunophenotyped using FACS. We also investigated the impact of sensory neuron silencing in a Th1-skewed model of allergic airway inflammation (Bogaert et al., 2011) following subcutaneous (s.c.) sensitization with OVA (1 mg/ml) in a 200- $\mu$ l emulsion of sterile PBS and 50% CFA on day 0 and with 50% incomplete Freund's adjuvant (IFA) on day 7. Another type 2 inflammation model was induced by HDM (Johnson et al., 2004). Lightly anesthetized (isoflurane) mice were sensitized (days 1–5) and challenged (days 8–10) with HDM (25  $\mu$ g/10  $\mu$ l, intranasally) and exposed to QX-314 (100  $\mu$ M, day 12). Mice were sacrificed on day 15 and BALF was harvested; cells were isolated, counted, and immunophenotyped using FACS.

## Statistics

Data are expressed as mean  $\pm$  SEM from 4–36 mice. Statistical significance determined by two-tail (unpaired or paired [patch-clamp]) Student's *t* test; *p* values less than 0.05 were considered significant. Numbers of animals are defined in figure legends.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2015.06.007>.

## AUTHOR CONTRIBUTIONS

S.T., R.-E.E.A., P.R.B., S.L., S.J.F.C., M.A.P., C.L., S.L.F., J.V.T., N.L., I.M.C., M.D., and B.A. conducted the experiments. C.V.H., O.H., and H.S. initiated the study. S.T., R.-E.E.A., P.R.B., C.L., M.A.P., S.L.F., N.G., D.R., I.M.C., V.K.K., B.P.B., B.D.L., and C.J.W. designed the study. S.T., J.M.P., V.K.K., B.P.B., B.D.L., and C.J.W. wrote the manuscript.

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